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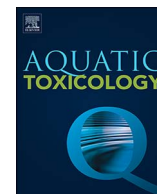
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Research paper

Sub-lethal effects of waterborne exposure to copper nanoparticles compared to copper sulphate on the shore crab (*Carcinus maenas*)



Lisa M. Rossbach, Benjamin J. Shaw, Dawid Piegza, William F. Vevers, Andrew J. Atfield, Richard D. Handy*

School of Biological and Marine Sciences, Plymouth University, Drake Circus, Plymouth PL4 8AA, United Kingdom

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ABSTRACT

The toxicity of soluble copper (Cu) to marine organisms is reasonably well described. However, the hazard of Cu engineered nanomaterial (ENMs) is poorly understood. The aim of the present study was to compare the toxicity of Cu ENMs to Cu as CuSO₄ in the shore crab, *Carcinus maenas*. The crabs were exposed via the water using a semi-static approach to 0.2 or 1 mg L⁻¹ of Cu ENMs or 1 mg L⁻¹ of Cu as CuSO₄. Gills, hepatopancreas, chela muscle and haemolymph were collected at days 0, 4 and 7 for the body burden of Cu, histology and biochemical analysis [thiobarbituric acid reactive substances (TBARS) and total glutathione (GSH)]. Nominal exposure concentrations of both the ENMs and the metal salt were maintained at over 80% in each treatment throughout the experiment. By day 7, 54% mortality was recorded in the 1 mg L⁻¹ CuSO₄ treatment, compared to just 21% in the 1 mg L⁻¹ Cu ENM-exposed crabs. The target organs for Cu accumulation were similar for both forms of Cu with highest concentrations in the gills, particularly the posterior gills; followed by the hepatopancreas, and with the lowest concentrations in the chela muscle. No changes were observed in the osmolality of the haemolymph (ANOVA, $P > 0.05$). TBARS were measured as an indicator of lipid peroxidation and showed the greatest change in the anterior and posterior gills and hepatopancreas of animals exposed to 1 mg L⁻¹ Cu ENMs (ANOVA or Kruskal-Wallis, $P < 0.05$). No statistically significant changes in total GSH were observed (ANOVA, $P > 0.05$; $n = 6$ crabs per treatment). Histological analysis revealed organ injuries in all treatments. The types of pathologies observed in the Cu ENM treatments were broadly similar to those of the Cu as CuSO₄ treatment. Overall, the target organs and Cu accumulation from Cu ENMs were comparable to that following exposure to Cu as CuSO₄, and although there were some differences in the sub-lethal effects, the metal salt was more acutely toxic.

1. Introduction

Emerging metal-containing engineered nanomaterials (ENMs) including Cu ENMs, pose new challenges to environmental protection (Handy et al., 2008; Klaine et al., 2008; Shaw and Handy, 2011; Klaine et al., 2012). Nanomaterials form colloidal dispersions in water and the key aspects of the water chemistry that influences ENM aggregation include the pH, ionic strength, presence of divalent ions such as calcium, and the type of organic matter (see Handy et al., 2008). For metal-containing ENMs, the dissolution of toxic free metal ions from the surface of the material is also a concern (Shaw and Handy, 2011; Albairuty et al., 2016). However, most of the existing data on the fate, behaviour and ecotoxicity of ENMs has been conducted on freshwater species, especially the organisms used in regulatory toxicity testing (reviews: Handy et al., 2008; Klaine et al., 2008; Kahru and

Dubourguier, 2010; Klaine et al., 2012). In contrast, the data set on marine species is lacking (Buffet et al., 2011; Matranga and Corsi, 2012).

In seawater, the issue of ENM aggregation at high ionic strength is a main concern as this raises the possibility of increased exposure of benthic organisms due to particles settling from the water column (Klaine et al., 2008). Consequently, the few studies with ENMs on marine organisms have focussed on filter feeding bivalves (e.g., *Mytilus* species, Gomes et al., 2014) and sediment dwelling organisms, (e.g., polychaete worms, Galloway et al., 2010). For many marine species, and even for entire taxonomic groups, ecotoxicity data are not available (Baker et al., 2014). Notably, decapod crustaceans appear to have received little attention. One approach to deal with the data gaps for a current environmental risk assessment of ENMs in marine systems would be to estimate the hazard from data sets on freshwater

* Corresponding author.

E-mail address: rhandy@plymouth.ac.uk (R.D. Handy).

organisms. In general, the acute toxicity values for metallic ENMs are in the μg to low mg L^{-1} range for freshwater species (*Daphnia*, Lovern and Klaper, 2006; fishes, Handy et al., 2011; zebrafish, Griffith et al., 2008; Shaw et al., 2016; rainbow trout, Shaw et al., 2012). For Cu ENMs, the target organs in freshwater fishes are known and similar to that of the dissolved metal counterpart (Griffith et al., 2008; Shaw et al., 2012); although the extent of sublethal effects may be different for the nanoparticulate form (e.g., organ pathologies, Al-Bairuty et al., 2013). However, correction factors for salinity effects on ENM toxicity are not available, and so such data would be used with uncertainty.

An alternative approach to hazard assessment of ENMs in marine systems would be to extrapolate from traditional data on dissolved metal toxicity in seawater. The effects of dissolved copper (Cu) on marine invertebrates has been extensively studied (e.g., Luoma and Rainbow, 2005; Rainbow, 2007). In seawater, dissolved Cu toxicity is partly ameliorated by the presence of high concentrations of NaCl and is explained by competition between dissolved Cu species and Na^+ for uptake by the animals (Grosell et al., 2007). Nonetheless, dissolved Cu can be acutely toxic to marine species in the range of a few hundred $\mu\text{g L}^{-1}$ to low mg L^{-1} concentrations (Schmidt and Foster, 1977; Bruland and Lohan, 2003; Grosell et al., 2007). LC_{50} values have been recorded as high as 2 mg L^{-1} in the shore crab *Carcinus maenas* following 6 days of exposure to Cu (Nonnotte et al., 1993). For waterborne exposure to dissolved Cu, the target organs in *C. maenas* include the gills, which show Cu accumulation with subsequent disruption of osmoregulation and/or cardio-respiratory functions (e.g., Weeks et al., 1993). Dissolved copper is taken up by the internal organs; with *C. maenas* showing accumulation in the hepatopancreas, carapace, heart and hypodermis (Bjerregaard and Vislie, 1986), and sometimes the muscle (Weeks et al., 1993). However, whether or not nano forms of Cu cause the same toxicity and target organ effects in crabs is unknown.

The aim of the present study was to determine the sublethal toxicity and target organs for Cu accumulation in *C. maenas* exposed to Cu ENMs compared to Cu as CuSO_4 . The approach here, similar to our previous work on fishes (e.g., Shaw et al., 2012), was to take a holistic view of possible sublethal physiological effects on the crabs. Consequently end points were selected that informed on physiological functions such as osmoregulation (e.g., tissue electrolytes, haemolymph osmolality), respiration (gill histopathology) and biochemical parameters associated with oxidative stress (thiobarbituric acid reactive substances, TBARS, and total glutathione, GSH, concentrations).

2. Materials and methods

2.1. Experimental design

Pre-moult shore crabs (*Carcinus maenas*) were collected from the estuary of the river Avon, Devon, UK, and allowed to acclimate for 5 days in a recirculation system containing 37‰ Plymouth Sound seawater at 13°C . Animals weighing $57.9 \pm 1.7 \text{ g}$, with a total length (posterior to anterior tip of the eyes) of $4.9 \pm 0.1 \text{ cm}$ and a carapace width of $6.2 \pm 0.1 \text{ cm}$ (data mean \pm SEM, $n = 144$), were randomly transferred to 12 experimental glass tanks (12 crabs per tank, 3 tanks per treatment, totalling 36 crabs/treatment) containing 20 L of seawater. The crabs were then allowed to rest for 24 h prior to the experiment. The animals were exposed in triplicate for 7 days using a semi-static exposure regime to one of the following treatments: control (no added Cu or Cu ENMs), $200 \mu\text{g L}^{-1}$ Cu ENMs, 1 mg L^{-1} Cu ENMs or 1 mg L^{-1} Cu (as CuSO_4). The Cu ENM concentrations were chosen after considering our previous finding on the Cu ENM dose need to cause measurable Cu accumulation and sub-lethal effects in trout (Shaw et al., 2012). The CuSO_4 was selected to act as a reference for comparison with the highest test concentration (1 mg L^{-1}) of the ENM and to contrast the hazard of the metal salt with the nanomaterial form. The crabs were not fed during the study to avoid Cu uptake through the diet and also to help maintain water quality. Water changes (80%) were

performed every 12 h with subsequent redosing (80% of the nominal concentration).

Water samples were taken before and after each water change in order to assess pH, dissolved oxygen (D.O.), temperature (all with a Hach HQ40d multi reader), salinity (hand held refractometer), Na^+ and K^+ (Flame Photometry, Corning 400), Ca^{2+} and Mg^{2+} (flame atomic absorption spectroscopy, F-AAS, Varian Spectra A 50B), and Cu, Zn and Fe (inductively couple plasma mass spectrometry, ICP-MS, xSeries 2, Thermo Scientific). Water quality parameters did not show any statistically significant differences between tanks (ANOVA, $P > 0.05$) and so data were pooled giving (grand mean \pm SEM, $n = 14$): temperature, $13.3 \pm 0.9^\circ\text{C}$; salinity, $39 \pm 0.3\text{‰}$; D.O., $6.3 \pm 1.7 \text{ mg L}^{-1}$ and pH, 7.3 ± 0.2 . Electrolyte concentrations were: Na^+ , $382.0 \pm 2.9 \text{ mmol L}^{-1}$; K^+ , $9.4 \pm 0.2 \text{ mmol L}^{-1}$; Zn, $0.7 \pm 0.2 \mu\text{mol L}^{-1}$; Fe, $0.9 \pm 0.2 \mu\text{mol L}^{-1}$; Mg, $4.9 \pm 0.2 \mu\text{mol L}^{-1}$ and Ca, $70.4 \pm 17.7 \mu\text{mol L}^{-1}$. The photoperiod was 12 h light: 12 h dark. Two crabs for each endpoint per tanks were randomly selected and sampled on days 0, 4 and 7 for tissue and haemolymph trace element concentrations, biochemistry and histology (the latter at day 7 only).

2.2. Stock dispersions, characterisation and dosing

The characterisation of the specific batch of Cu ENMs used in the study has already been reported (see Shaw et al., 2012 for details). The Cu ENMs were purchased as a metal powder from Sigma Aldrich (manufacturer's information: 99.9% purity, $< 50 \text{ nm}$ particle size). The mean primary particles size of the material was determined by transmission electron microscopy (TEM, JEOL 1200EXII) at Plymouth University, along with the hydrodynamic diameter of the dispersion in ultrapure water measured using Nanoparticle Tracking Analysis (NTA, NanoSight LM10). The values were $87 \pm 27 \text{ nm}$ (mean \pm SD, $n = 50$ particles) for primary particle diameter and $216 \pm 122 \text{ nm}$ (mean \pm SD, $n = 6$ different sub-samples of the stock) for the hydrodynamic diameter in ultrapure water respectively (Shaw et al., 2012).

In addition, attempts were made to characterise the Cu ENMs in seawater for the present study (see Supplementary Fig. S1). As expected, the natural Plymouth seawater used in the experiments had a background of colloids that was detectable; with a particle number concentration around $0.6 \times 10^6 \text{ mL}^{-1}$ and a mean hydrodynamic diameter of $265 \pm 14 \text{ nm}$ (mean and SD, $n = 3$ samples). This natural background effectively masked any attempt to quantify the Cu ENM dispersion in the natural seawater. However, instead, some measurements were made on 50 mg L^{-1} dispersions made in artificial seawater (Fig. S1). The artificial seawater contained (in mmol L^{-1}): 500, NaCl; 50, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 10, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 10, KCl and was adjusted to pH 7.3 to match the aquarium seawater. The response in artificial seawater was bimodal with peaks at 80 nm and 210 nm, representing the presence of dispersed primary particles and aggregates respectively (Fig. S1).

The dissolution rate of the Cu ENMs was also measured by dialysis in artificial seawater to determine the maximum theoretical dissolved Cu release over a typical 12 h water change used in the experiments. The dialysis method was based on Besinis et al. (2014). Briefly, acid-washed then deionised glassware was used throughout. A 50 mg L^{-1} stock was prepared in ultrapure (Milli-Q) water and sonicated for 4 h at room temperature (bath type sonicator, 35 kHz frequency, Fisherbrand FB 11010, Germany). Then, 10 mL of the dispersion was added to dialysis tubing (molecular weight cut off at 12,000 Da, Sigma-Aldrich, UK) cut to size and sealed. The dialysis bag containing the Cu ENM was placed in a beaker containing 490 mL of artificial seawater (recipe above, performed in triplicate) and left stirring gently at room temperature for 12 h. The control was the dialysis bags without added Cu ENMs. Seawater samples were taken at time zero and 12 h, acidified immediately with a few drops of 5% HNO_3 and subsequently diluted 1:4 with 5% HNO_3 total Cu determination (see below). The measured total dissolved Cu concentration in the beakers after 12 h was $92 \pm 1.77 \mu\text{g L}^{-1}$ (mean \pm S. D., $n = 3$ beakers); equating to a total

dissolution of 46 µg of Cu per beaker, or 9.2% of the Cu ENM over 12 h.

For the main experiments with the crabs, a 50 mL stock solution of 4 g L⁻¹ Cu ENMs was prepared daily in order to minimise the risk of Cu dissolution over time in the experiment. Briefly, the ENMs were added to Milli-Q water (ultrapure, 18.2 MΩ-cm resistivity, ion free and unbuffered) without solvents and sonicated (bath type sonicator, 35 kHz frequency) for 4 h. A 1 mg L⁻¹ Cu (as CuSO₄) stock solution was prepared by adding 7.85 g of CuSO₄·5H₂O (Sigma reagent Plus, ~99%) to 500 mL of Milli-Q water and stirred (magnetic stirrer IKA Werke RET basic C, at 300 rpm) for 30 min. The same CuSO₄ stock solution was used throughout the 7 day exposure period.

2.3. Dissection and analysis of haemolymph

Only surviving animals were used for the collection of haemolymph and tissues. Crabs were removed individually from each tank and haemolymph samples taken between the rear and the fourth walking leg using a syringe. These samples were stored in cryotubes at -80 °C until required for analyses. Whole haemolymph samples were analysed for Ca²⁺ and Mg²⁺ using flame atomic absorption spectrometry (F-AAS, Varian Spectra A 50B), and Na⁺ and K⁺ using a flame photometer (Corning). The osmolarity of the haemolymph (20 µL samples) was determined using an Osmomat 030 Cryoscopic osmometer with readings taken against 1000 mOsmkg⁻¹ standards provided by the manufacturer.

Prior to removal of the shell and subsequent dissection, the animals were euthanised by cutting the central ganglion. Gills (posterior and anterior), the hepatopancreas and chela muscle were carefully dissected with clean, ion free instruments and rinsed using MilliQ water before processing for metal ions analysis according to Handy et al. (1999), with modifications for Cu ENMs (Shaw et al., 2012). Tissue samples (as above) for the biochemical analysis were carefully removed from a further two crabs from each tank, rinsed and placed in cryogenic tubes, snap frozen in liquid nitrogen and then stored at -80 °C. Histological samples were also taken at day 7 (end of the exposure) from a further two crabs per tank.

2.4. Metal analysis

Tissue samples from surviving crabs were analysed for ions using a modification of the method used by Shaw et al. (2012). Briefly, wet tissues were oven dried to a constant weight (60 °C for 48 h) and dry weights (dw) recorded. The dried tissue samples (typically 0.1–0.3 g dw) were transferred into 15 mL polypropylene copolymer tubes (Elkay, Basingstoke, UK) and digested in 3 mL concentrated analytical grade nitric acid (69% analytical grade, Fisher Scientific) in a water bath at 75 °C for 10 h. Samples were then cooled and diluted with 12 mL ultrapure water. The tissue digests were analysed for Na⁺, K⁺, Ca²⁺ and Mg²⁺ as described above for haemolymph (i.e., F-AAS and flame photometry). Tissue Cu, Fe, Zn and Mn were determined by inductively coupled plasma mass spectrometry (ICP-MS) against mixed, matrix matched standards (0–100 µg L⁻¹) prepared from certified Aristar plasma emission grade solutions. Quality control was assured by carrying out accuracy checks using a known standard or blank every 10 samples during the analysis. Also, 2% internal iridium and indium standards (P/N/4400-013 CPI, Quality control standard 26) were added to each sample. DORM-3 (dogfish certified reference material – CRM) from National Research Centre Canada (NRCC) was used to verify the digestion procedure for Cu metal, giving a measured total Cu of 14.72 ± 2.02 µg g⁻¹ dw (mean and S.D., n = 3 digestions), which was not statistically different from the expected reference value of 15.5 ± 0.63 µg g⁻¹ dw. Due to the lack of appropriate reference materials for Cu ENMs, a spike recovery test was conducted to determine the percentage recovery of nanoparticles in crab tissue and water samples by ICP-MS. Percentage recoveries of Cu ENMs for the gills, hepatopancreas and the muscle tissue were 78.2 ± 41.4, 86.8 ± 12.7

and 60.0 ± 4.6% (mean ± S.E.M., n = 6) respectively.

2.5. Biochemistry

The biochemical analyses of tissues from surviving crabs was conducted according to Smith et al. (2007). Briefly, tissues were homogenized (Cat X520D with a T6 shaft, medium speed, Bennett & Co., Weston Super-Mare) in 2.5 mL In artificial seawater, the overall mean hydrodynamic diameter was 243 ± 43 nm (Fig. of ice-cold isotonic buffer (300 mmol L⁻¹ of sucrose, 0.1 mmol L⁻¹ of EDTA (Ethylene-diaminetetraacetic acid) and 20 mmol L⁻¹ of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) dissolved in MilliQ water (pH 7.8 adjusted using a few drops of 2 M Tris (2-amino-2-hydroxymethyl-1,3-propanediol)). Homogenates were stored at -80 °C until required. Tissues were subsequently defrosted on iced water and analysed for total glutathione (GSH, 20 µL of homogenate) and thiobarbituric acid reactive substances (TBARS, 40 µL of homogenate). TBARS data were normalised per mg of homogenate protein, whilst GSH was expressed per g wet weight tissue. For protein determination, a Pierce BCA Protein Assay Kit was used and 30 µL of homogenate, defrosted from anterior and posterior gills, hepatopancreas and muscle tissue for the determination of protein concentrations. All samples were read on a Versa Max microplate reader in 96 well microplates.

2.6. Histology

For the surviving animals, two crabs from each test vessel (6 per treatment) were collected for histology. The anterior and posterior gills, hepatopancreas and muscle tissue, were carefully collected at the end of the experiment and fixed in 4% formalin prepared in seawater. Samples were dehydrated in alcohol, cleared in xylene and manually set in paraffin wax blocks. Haematoxylin and eosin (Mayer's H & E) was used for the staining of the 7 µm thick sections which were examined by light microscopy using an Olympus Vanox-T microscope. Images were taken using an Olympus camera C-2020 Z camera. The lamellar width (LW) and the haemolymph space diameter (HSD) of the gill filaments in anterior and posterior gills were measured manually on the collected images using ImageJ software (1.45 s). Briefly, measurements were made on ten separate lamellae from the middle portion of each gill arch in each crab, and an average value (technical replicate, within gill sample) determined for the animal. Treatment means and standard errors (between crab variation within treatment) were then calculated from the average morphometrics of each of the six animals examined per treatment.

2.7. Statistical analysis

All data were analysed using StatGraphics Centurion (XVI). Values were considered to be significantly different at a P-value equal or lower than 0.05 at a 95% confidence limit. Following descriptive statistics and an assessment of unequal variance (Bartlett's test), a multifactor ANOVA was employed to test for treatment, time and treatment x time interactions. The Fisher's 95% least-square difference post hoc test was used to identify the location of statistical differences. Where the multifactor ANOVA indicated significant differences, a one-way ANOVA was used to assess for simple effects. Where data were non-parametric and could not be transformed, the Kruskal-Wallis test was used to test for time or treatment-effects and differences located using notched box and whisker plots.

3. Results

3.1. Waterborne exposure to Cu ENMs and Cu as CuSO₄

Copper exposure was confirmed by ICP-MS analysis of water samples taken 10 min post-dosing and showed good recovery (all measured

Table 1

Measured total copper concentrations in the experimental tanks of shore crabs exposed to Cu as CuSO₄ or as Cu ENMs in full strength seawater.

Treatment	10 min post-dosing		12 h post-dosing	
	Measured concentration (mg L ⁻¹)	% of nominal	Measured concentration (mg L ⁻¹)	% of nominal
Control	< 0.001	NA	< 0.001	NA
200 µg L ⁻¹ Cu ENM	0.177 ± 0.019	88.3 ± 9.4	0.161 ± 0.015	80.6 ± 7.7
1 mg L ⁻¹ Cu ENM	0.855 ± 0.060	85.5 ± 6.0	0.841 ± 0.047	84.1 ± 4.7
1 mg L ⁻¹ CuSO ₄	1.192 ± 0.064	119.2 ± 6.4	1.082 ± 0.035	108.2 ± 3.5

A semi-static exposure regime was used, with water changes every 12 h. Water samples were taken 10 min and 12 h after the dosing of experimental tanks. Data are mg L⁻¹, mean ± SEM, *n* = 12 water samples per treatment. No significant differences were observed between triplicate tanks (ANOVA, *P* > 0.05), therefore data were pooled by treatment. NA = not applicable.

values were over 80% of the nominal concentrations, Table 1). Further water samples taken 12 h after the experimental tanks were dosed confirmed that the crabs were exposed to over 80% of the nominal concentrations throughout the duration of the 12 h exposures periods between water changes with no significant differences within treatments over time (ANOVA, *P* > 0.05, Table 1). Mortality was seen following exposure to both the 1 mg L⁻¹ Cu ENMs and Cu (as CuSO₄) treatments (Fig. 1). The metal salt was more acutely toxic with 50% mortality (LT₅₀) reached after 6.5 days, compared to only 21% mortality in the 1 mg L⁻¹ Cu ENMs after 7 days (Fig. 1). There were no mortalities in either the control crabs or the 200 µg L⁻¹ Cu ENM treatment.

Copper accumulation was observed in the anterior and posterior gills, and the hepatopancreas and muscle following exposure to both Cu ENMs and Cu as CuSO₄, compared to unexposed controls (Fig. 2). For animals exposed to CuSO₄, as expected, the gills showed elevations of total Cu with exposure time. There were no differences in the total Cu concentration between the anterior and posterior gill in the CuSO₄-exposed animals at day 4 or day 7 (ANOVA, *P* > 0.05). In contrast, a statistically significant increase in total Cu concentration was seen in

the anterior gill compared to the posterior gill of crabs exposed to 1 mg L⁻¹ Cu ENMs at day 4 (1139 ± 108 and 861 ± 45 µg g⁻¹ respectively, ANOVA, *P* = 0.038, Fig. 2), though this effect was lost by day 7 (total Cu in the anterior and posterior gills was 1226 ± 130 and 1289 ± 194 µg g⁻¹ respectively, ANOVA, *P* = 0.749). However, after 7 days of exposure to Cu ENMs, the total Cu concentrations in the gill tissue was lower than in the CuSO₄ exposed crabs (Fig. 2), regardless of whether the tissue was anterior or posterior gill. For the ENM-exposed animals, statistically significant increases in total Cu concentration within the hepatopancreas was observed after 7 days of exposure compared to unexposed controls (ANOVA, *P* < 0.05), with significantly higher concentrations of Cu resulting from exposure to the Cu salt at both time points (Fig. 2c, ANOVA, *P* < 0.05). In the muscle tissue a significant increase in Cu was seen in all treatments compared to control crabs at day 4, but with no differences between the Cu materials themselves (Cu ENMs versus Cu salt, Fig. 2d, ANOVA, *P* < 0.05). However, by day 7 Cu levels in the muscle of crabs from both ENM treatments had decreased to control levels, and only the crabs exposed to CuSO₄ showed elevated Cu in the muscle at the end of the experiment. There were no statistically significant differences in Cu concentration within the haemolymph for any treatment compared to controls (ANOVA, *P* > 0.05). The only difference in Cu levels in the haemolymph over time was seen in the CuSO₄ treatment, with a significant elevation between days 4 and 7 (Fig. 2e, ANOVA, *P* < 0.05).

3.2. Tissue ion concentration and haemolymph osmolarity

Overall, only minor changes were seen in the tissue electrolyte composition of exposed crabs (Table 2). A statistically significant decrease in Na⁺ levels was observed over time for all tissues examined as well as the haemolymph (ANOVA, *P* < 0.05). An assessment of K⁺ levels within anterior and posterior gills, hepatopancreas and muscle showed no significant differences (ANOVA, *P* > 0.05) and concentrations remained fairly stable throughout the exposure period (Table 2). A significant depletion of Fe from the hepatopancreas was seen over time (ANOVA, *P* < 0.05) in samples taken from 1 mg L⁻¹ Cu ENM exposed crabs compared to other treatments. Neither Zn (Table 2) nor Mn (data not shown) showed any significant treatment dependent effects, with values ranging between 1.4–9.9 and 0.1–3.0 µmol g⁻¹ dry weight tissue respectively depending on the tissue analysed (i.e., gills, hepatopancreas or muscle). For instance concentrations of Mn in the anterior gill

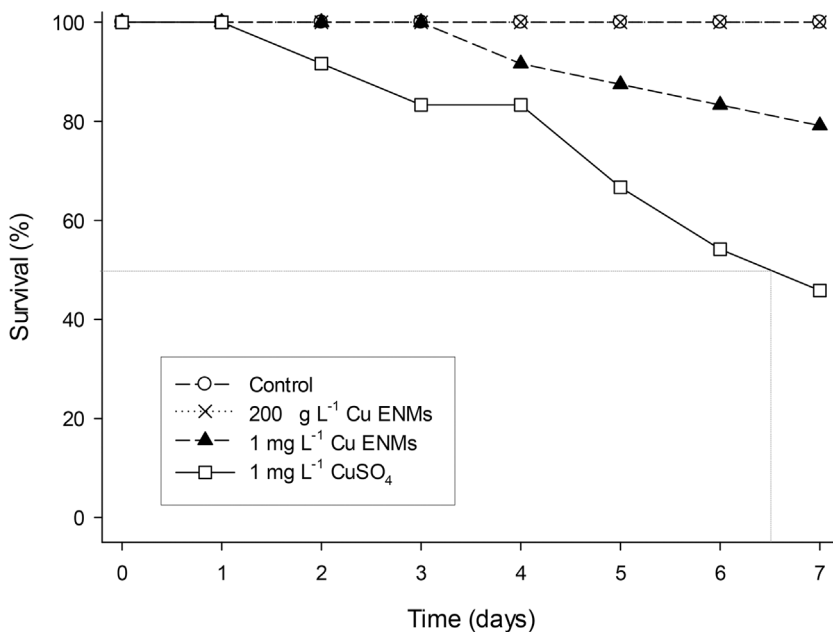


Fig. 1. Lethal-time curve showing the percent survival of shore crabs within different treatments (control, 200 µg L⁻¹ Cu ENMs, 1 mg L⁻¹ Cu ENMs and 1 mg L⁻¹ CuSO₄) following 7 days of exposure. LT₅₀ for CuSO₄ were recorded after 6.5 days (indicated by light grey dashed line). Error bars are omitted for clarity.

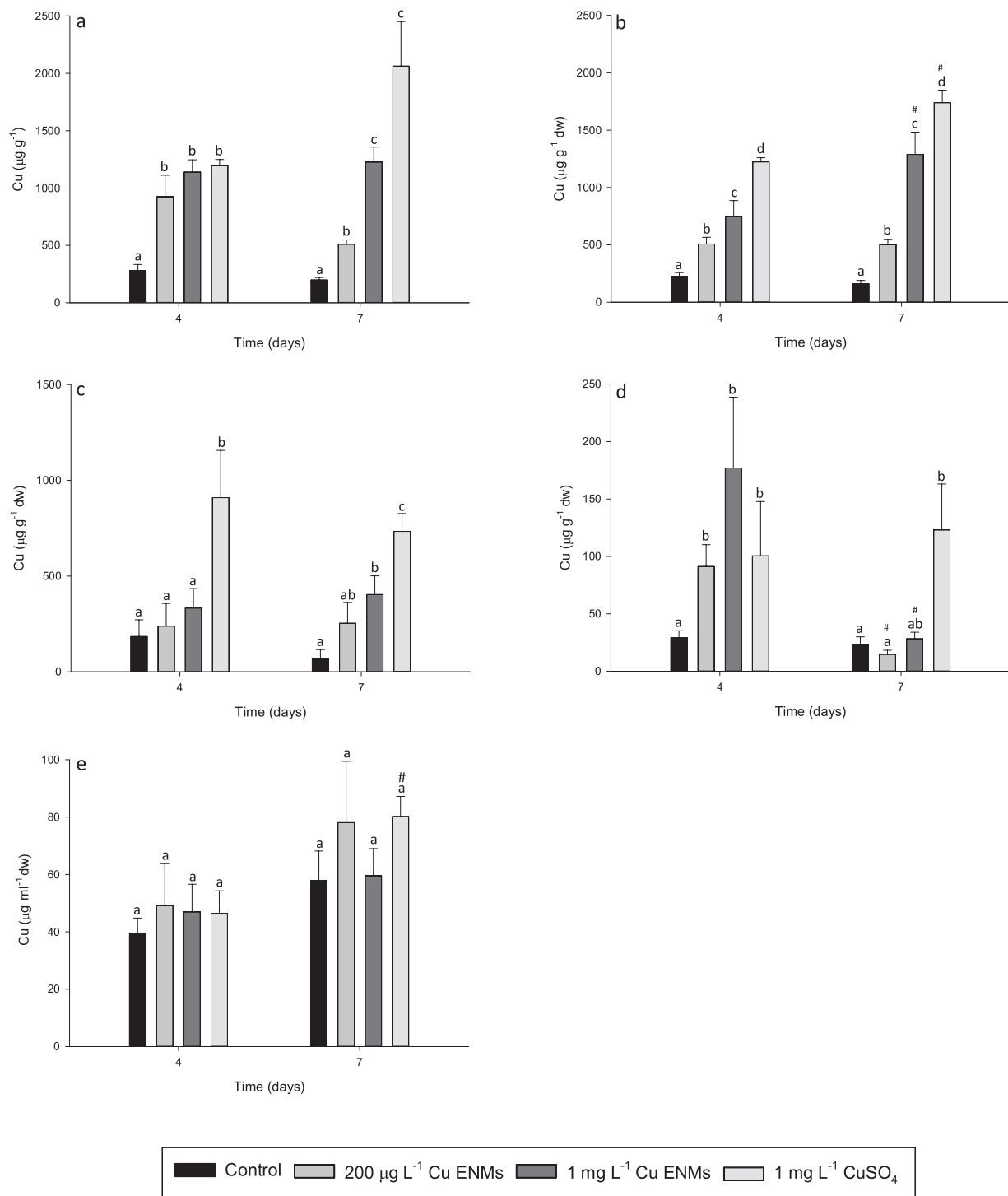


Fig. 2. Total Cu concentration ($\mu\text{g g}^{-1}$ dry weight except haemolymph in $\mu\text{g ml}^{-1}$) in tissues of the shore crab; anterior gills (a), posterior gills (b), hepatopancreas (c), muscle (d) and haemolymph (e) at days 4 and 7 of exposure. Data are mean \pm S.E.M., $n = 6$ crabs per treatment. Different letters indicate statistically significant differences within a time point (ANOVA, $P < 0.05$); # indicates time dependent significant differences within a treatment between days 4 and 7 (ANOVA, $P < 0.05$). Initial stock crabs at time zero are not shown for clarity, but background Cu concentrations in the stocks were (data; mean \pm S.E.M., $\mu\text{g g}^{-1}$ dw); anterior gills (289 ± 97), posterior gills (353 ± 102), hepatopancreas (233 ± 142), muscle (56 ± 49) and haemolymph ($69 \pm 29 \mu\text{g ml}^{-1}$).

for control, $200 \mu\text{g L}^{-1}$ and 1 mg L^{-1} Cu ENMs, and 1 mg L^{-1} CuSO₄ (mean \pm SEM, $n = 24$) were 0.2 ± 0.1 , < 0.1 , 0.3 ± 0.1 and $0.3 \pm 0.1 \mu\text{mol g}^{-1}$ respectively. Osmolarity in haemolymph samples did not show much variation from the mean value (mean \pm SEM, $n = 53$) of $963 \pm 2.3 \text{ mOsm L}^{-1}$ with no statistically significant differences compared to controls (ANOVA, $P > 0.05$).

3.3. Biochemistry

TBARS within the anterior (Fig. 3a) and posterior gills (Fig. 3b) showed no statistical differences compared to controls, regardless of the type of Cu exposure by the end of the experiment. However, there was a transient rise in TBARS in the anterior gills of crabs exposed to 1 mg L^{-1} Cu as the ENM at day 4, reaching $25.2 \pm 6.8 \text{ nmol mg}^{-1}$

Table 2Trace element concentrations in the tissues and haemolymph of shore crabs exposed Cu ENMs or Cu as CuSO₄ in full strength seawater.

Tissue	Treatment	Concentration			
		Na ⁺ (mmol g ⁻¹ dw)	K ⁺ (mmol g ⁻¹ dw)	Fe (μmol g ⁻¹ dw)	Zn (μmol g ⁻¹ dw)
Anterior Gills	Control	293.2 ± 108.4	31.2 ± 1.8	9.8 ± 2.6	2 ± 0.2
	200 μg L ⁻¹ Cu ENM	179.7 ± 16.0	33.7 ± 1.6	17 ± 6.3	2.1 ± 0.2
	1 mg L ⁻¹ Cu ENM	189.4 ± 13.5	26.8 ± 3.0	23.9 ± 27.2	2 ± 0.2
	1 mg L ⁻¹ CuSO ₄	240.5 ± 27.7	29.2 ± 2.8	11.9 ± 3.9	2.4 ± 0.4
Posterior Gills	Control	143.7 ± 26.2	33.3 ± 6.2	7.4 ± 3.4	1.5 ± 0.3
	200 μg L ⁻¹ Cu ENM	191.6 ± 17.1	37.6 ± 2.8	11.9 ± 5.9	2 ± 0.1
	1 mg L ⁻¹ Cu ENM	211.1 ± 23.8	31.7 ± 2.6	16.3 ± 8	2.2 ± 0.2
	1 mg L ⁻¹ CuSO ₄	224.9 ± 26.8	32.7 ± 3.7	12.9 ± 4	2.8 ± 0.4
Hepatopancreas	Control	63.1 ± 13.7	37.1 ± 4.7	6.3 ± 0.8	2.3 ± 0.3
	200 μg L ⁻¹ Cu ENM	76.1 ± 23.3	49.2 ± 12.4	7.1 ± 1.4	2.1 ± 0.4
	1 mg L ⁻¹ Cu ENM	46.1 ± 10.2	32.2 ± 5.5	4.3 ± 0.8	2.2 ± 0.8
	1 mg L ⁻¹ CuSO ₄	45.8 ± 2.7	30.2 ± 2.8	5.9 ± 0.7	2.2 ± 0.3
Muscle	Control	60.1 ± 5.8	34 ± 1.3	0.6 ± 0.2	5.2 ± 0.5
	200 μg L ⁻¹ Cu ENM	74.2 ± 17.5	60.1 ± 20.7	0.6 ± 0.4	6.9 ± 0.4
	1 mg L ⁻¹ Cu ENM	50.6 ± 4.7	32.9 ± 1.5	0.6 ± 0.1	6.3 ± 0.2
	1 mg L ⁻¹ CuSO ₄	63.4 ± 6.5	39.1 ± 2.3	0.7 ± 0.2	5.5 ± 0.5
Haemolymph	Control	424.8 ± 6.4	10.2 ± 0.2	< 0.1	0.4 ± 0.1
	200 μg L ⁻¹ Cu ENM	357.8 ± 16.8	11.3 ± 0.9	< 0.1	0.5 ± 0.1
	1 mg L ⁻¹ Cu ENM	361.9 ± 49.3	11.6 ± 0.9	< 0.1	0.3 ± 0.0
	1 mg L ⁻¹ CuSO ₄	375.3 ± 19.2	11.3 ± 0.3	< 0.1	0.5 ± 0.1

Data are expressed g⁻¹ dry weight tissue, except for haemolymph which is mmol L⁻¹ (mean ± SEM, n = 24) following 7 days of exposure. No statistical differences were observed between treatments.

protein, but this effect was lost by day 7. There were no statistically significant changes observed in the TBARS of the hepatopancreas, with all treatments remaining similar to the controls (data not shown). Total glutathione (GSH) content was measured in anterior and posterior gills (Figs. 3c and d), and hepatopancreas (data not shown), with no treatment-dependent differences observed (ANOVA, P > 0.05). Total GSH remained stable for all treatments. For example, at the end of the experiment, GSH values in the anterior gills were (mean ± SEM, n = 6) 0.08 ± 0.00, 0.08 ± 0.00, 0.08 ± 0.00 and 0.08 ± 0.00 μmol g⁻¹ for control, 200 μg L⁻¹ and 1 mg L⁻¹ Cu NP and 1 mg L⁻¹ Cu as CuSO₄ respectively (Fig. 3c).

3.4. Histology

Gill pathology was almost absent in the control animals, with no evidence of oedema and only a background incidence of necrotic cells, although this is expected in animals collected from the wild (Fig. 4). In the 1 mg L⁻¹ Cu as CuSO₄ treatment, three out of six crabs showed fusion of lamellar tips with congestion of haemocytes in the anterior and posterior gill, with more severe effects observed in the latter. Five crabs displayed significant separation of epithelium at the lamellar tips and a thickening of epithelium of the middle part of the filaments; with necrosis or congestion of haemocytes and swollen mucocytes in the tissue (Kruskal-Wallis, P < 0.001, Fig. 4). Significant reductions of the haemolymph space diameter (HSD) and the increases in the lamellar width (LW) were found between 1 mg L⁻¹ Cu ENM and 1 mg L⁻¹ Cu as CuSO₄ exposed crabs (Kruskal-Wallis, P < 0.001, Fig. 4). Gill samples taken from 1 mg L⁻¹ Cu as CuSO₄ treatment showed more severe effects than that observed in the 1 mg L⁻¹ Cu ENM exposed crabs, with two of the former crabs displaying fusion of lamellar tips with congestion of haemocytes and frequent necrosis. Six animals from the 1 mg L⁻¹ Cu ENM treatment showed thickening of epithelium with frequent disruption of pillar cells and three crabs displayed significant separation of epithelium.

Despite some fixation artefacts in the hepatopancreas, samples taken from the 1 mg L⁻¹ Cu as CuSO₄ treatment showed discernible pathology, including the occasional loss of the epithelial integrity of the hepatopancreatic tubules. In other areas of the hepatopancreas where

the epithelium remained intact, the cells exhibited pyknosis and karyorrhexis. There was also an increased incidence of epithelial cells showing vacuoles, consistent with some lipid peroxidation. Muscle tissue taken from the same animals also showed signs of mild inflammation of myofibrils, including one animal with mononuclear cellular infiltrate in the interstitium. Hepatopancreas and muscle tissue collected from animals exposed to the 1 mg L⁻¹ Cu ENM treatment showed similar effects to those observed in samples taken from the 1 mg L⁻¹ CuSO₄ treatment (data not shown).

4. Discussion

This study is one of the first to compare waterborne exposure to Cu ENMs with that of CuSO₄ in the shore crab, *Carcinus maenas*. The results show that the metal salt is more toxic than the equivalent Cu ENM, but the target organs and patterns of total metal accumulation are broadly similar for both forms of Cu. The gills, as expected for waterborne exposures, were the primary target organ for both forms of the metal. However, there were some subtle differences in the organ pathologies of the animals, with the CuSO₄-treated animals being slightly more susceptible to gill injury and inflammation pathology.

4.1. Copper exposure

Exposure to both Cu ENMs and Cu as CuSO₄ was confirmed by analysis of water samples taken over the 12 h period between water changes. All the measured total Cu concentrations were over 80% of the nominal concentration in accordance with OECD regulatory toxicity guidelines (see Handy et al., 2012 on test methods). Some decrease in total Cu concentrations within the Cu ENM tanks was expected due to particle settling in the high ionic strength of seawater (Klaine et al., 2008). However this was not observed; suggesting that the aeration/incidental mixing of the water in the experimental tanks, along with the 12 h water changes were sufficient to maintain the exposure. The natural organic matter present in Plymouth seawater may have also aided the dispersion (as suggested for organic matter by Torres-Duarte et al., 2016 with CuO ENMs). Unfortunately, the interferences from the natural colloids in the Plymouth seawater prevented the use of

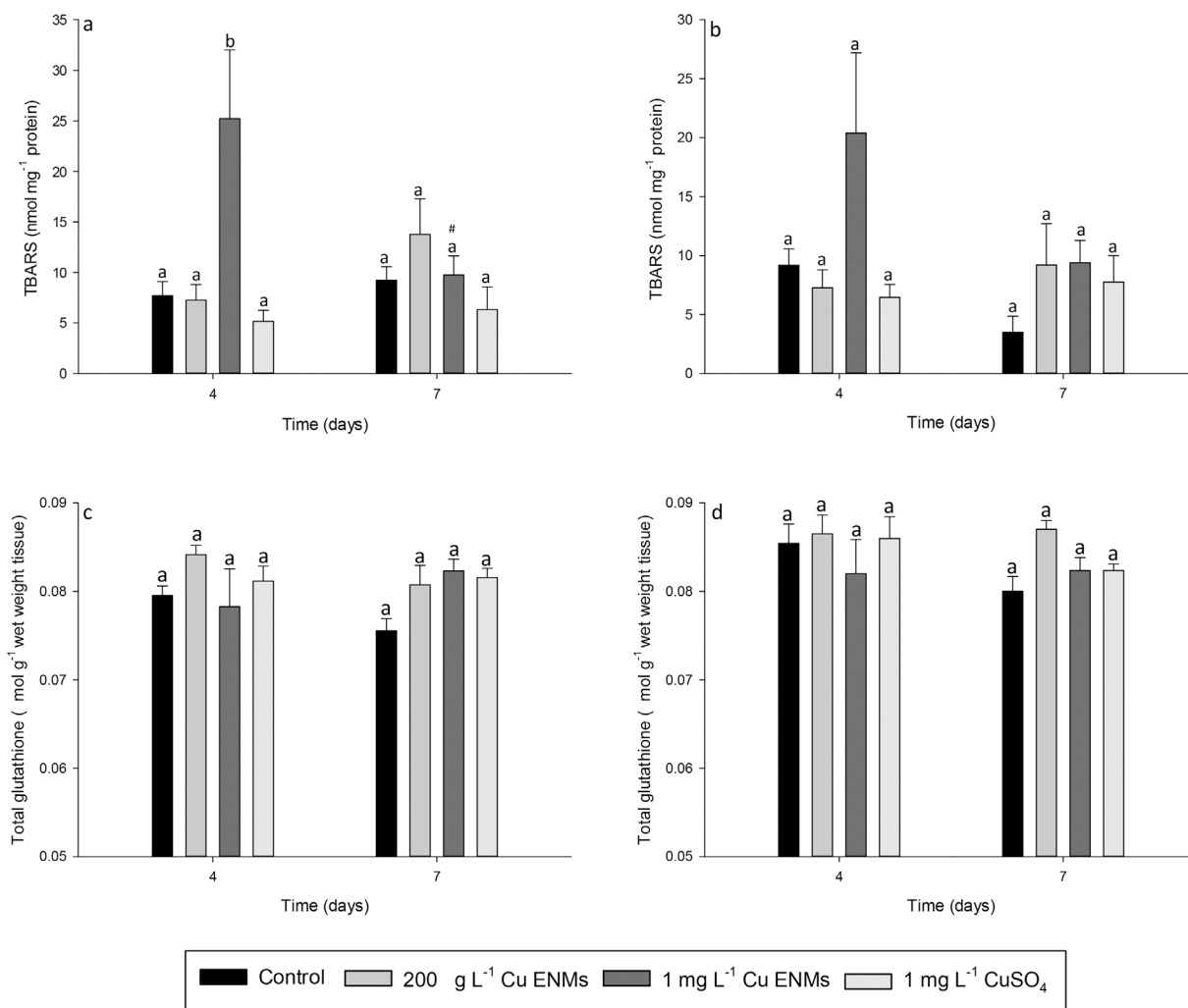


Fig. 3. Thiobarbituric acid reactive substances (TBARS, nmol mg^{-1} protein) in anterior (a) and posterior gills (b); and total glutathione ($\mu\text{mol g}^{-1}$ wet weight tissue) in the anterior (c) and posterior gills (d) of shore crabs exposed to control, $200 \mu\text{g L}^{-1}$ Cu ENMs, 1 mg L^{-1} Cu ENMs or 1 mg L^{-1} CuSO_4 for up to 7 days. Data are mean \pm S.E.M., $n = 6$ crabs per treatment). Different letters indicates statistically significant differences (ANOVA, $P < 0.05$), # indicates time dependent significant differences within a treatment between days 4 and 7 (ANOVA or Kruskal-Wallis, $P < 0.05$).

Nanoparticle Tracking Analysis (NTA) to follow the dispersion in the tanks. However, artificial seawater did show a bimodal particle dispersion that included primary particles (Fig. S1). Regardless, the Cu exposure was also confirmed by determining the total Cu concentrations in the tissues of exposed animals compared to controls (see below).

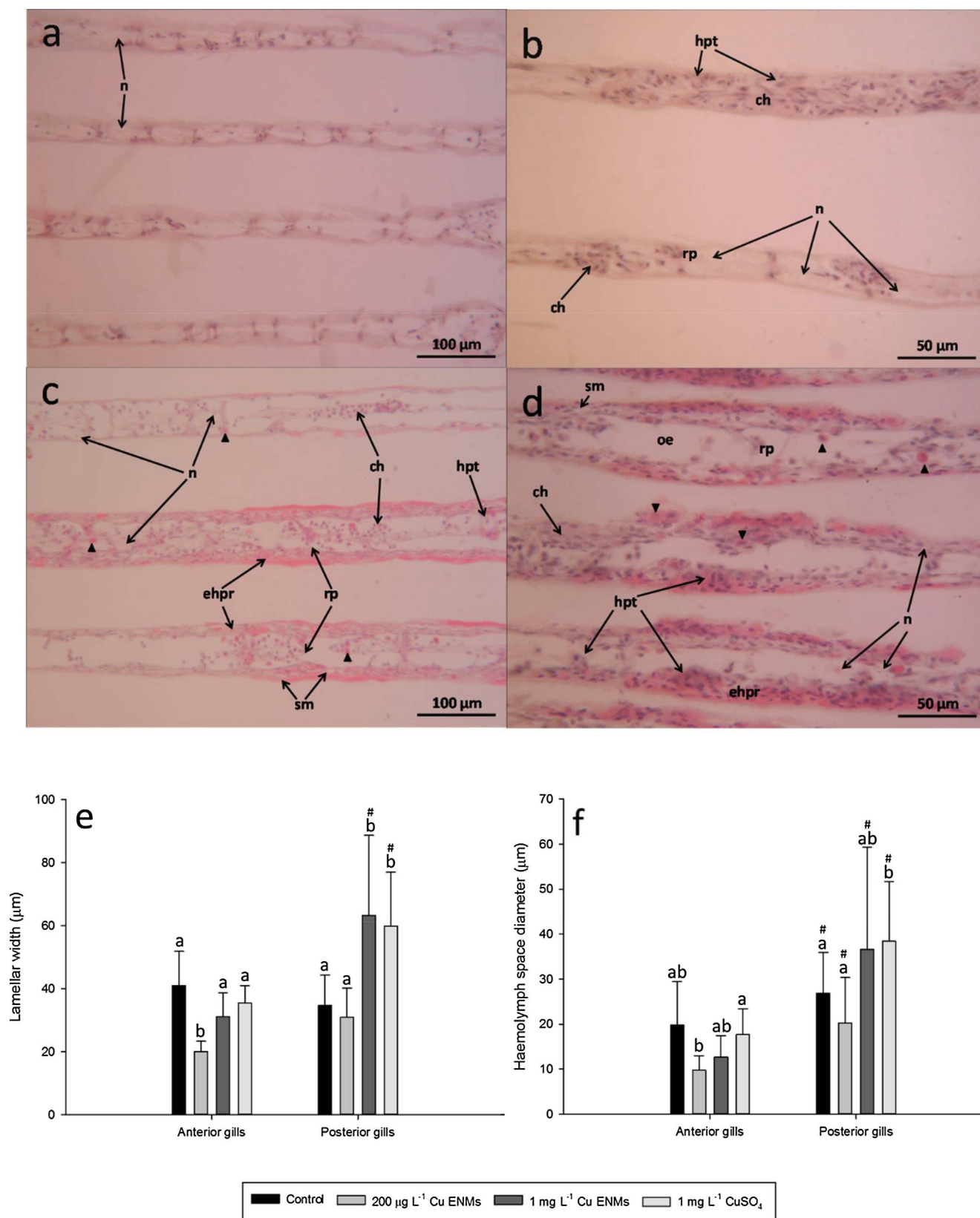
4.2. Copper accumulation and toxicity following waterborne exposure

The experiment aimed to achieve a progressive toxicity at the highest Cu concentration employed, so that metal accumulation in the tissues could be explored in context with other biological effects. All the control animals survived, as expected (Fig. 1), and the background total Cu concentrations in the tissues were consistent with previous reports of crabs held in seawater (~ 100 – $300 \mu\text{g g}^{-1}$ dw depending on organ, Rainbow, 1985; Truchot and Rtal, 1998). A progressive mortality was observed in the CuSO_4 -exposed crabs (LT_{50} of 6.5 days at 1 mg L^{-1} , Fig. 1). This is consistent with previously reported values of lethal toxicity to *Carcinus maenas* of around 1 – 2 mg L^{-1} of Cu over 6 days (Boitel and Truchot, 1989; Nonnotte et al., 1993). Crucially, at the same nominal concentration of 1 mg L^{-1} of Cu metal, the Cu ENM caused only 20% mortality by the end of the experiment. The toxicity of the Cu ENMs is not easily explained by the released of dissolved Cu species into the water, since the dissolution studies estimated only a 9% dissolution

of the ENM over the 12 h period leading to the next water change. Using the LT_{50} here (Fig. 1) as a bench mark for the metal salt hazard; the release of any dissolved Cu from the ENM might contribute at a maximum of only 5% of the observed mortality in the ENM treatment – the remaining being due to the nano form. It is also theoretically possible that an unknown mode of action of the Cu ENMs to cause lethal toxicity was ‘missed’ by the selected respiratory, osmoregulatory and oxidative stress endpoints used in this experiment.

However, the difference in toxicity between the metal salt and the ENM is better explained by lower metal accumulation in the gills of the animals exposed to the Cu ENM compared to those exposed to the Cu as CuSO_4 by the end of the experiment (Fig. 2); implying the nano form is less bioavailable. This pattern of higher accumulation from the Cu salt was reflected in the haemolymph and in the muscle tissue (day 7 only for the latter, Fig. 2) and occurred without statistical differences in the measured total Cu concentrations in the exposure tanks (Table 1). Interestingly, the Cu ENM also followed the notion of dose-response, since the Cu concentrations in the gill tissues at least, were lower in the $200 \mu\text{g L}^{-1}$ compared to 1 mg L^{-1} ENM exposure.

The gills of shore crabs are particularly interesting from an ecotoxicity perspective, because the anterior and posterior gills have different biological functions. The posterior gills are of great importance to ion transport, whilst the 4–6 anterior gill segments are primarily related to respiratory functions (review, Henry et al., 2012). In the



(caption on next page)

present study, the total Cu concentration in the anterior (respiratory) gill were significantly higher than those of the posterior (osmoregulatory) gill tissue in crabs exposed to 1 mg L^{-1} of Cu as Cu ENM, and although the difference was not statistically significant by the end

of the experiment; the observation indicates respiratory function as a target. Moreover, there were also differences in the types of gill pathology (see below).

Of the internal organs, the hepatopancreas is known to be a target

Fig. 4. Anterior gill morphology in shore crabs following 7 day exposure to (a) control, (b) $200 \mu\text{g L}^{-1}$ Cu ENMs, (c) 1 mg L^{-1} Cu ENMs or (d) 1 mg L^{-1} Cu as CuSO_4 . Panels (e) and (f) show anterior and posterior gill lamellar width (LW) and haemolymph space diameter (HSD) respectively. Different letters indicate a statistically significant difference in LW or HSD within either the anterior or posterior gills (ANOVA or Kruskal-Wallis, $P < 0.05$), whilst # indicates that there was a significant difference between the anterior and posterior gills within a treatment (ANOVA or Kruskal-Wallis, $P < 0.05$). Control gills (a) displayed normal histology with a background incidence of necrosis. Each of the Cu treatments (both nano and salt) had necrotic cells, but these were more prevalent in the 1 mg L^{-1} Cu treatments. Gills from the $200 \mu\text{g L}^{-1}$ Cu ENM treatment (panel b) experienced hypertrophy and congestions of haemocytes (the latter also seen in the 1 mg L^{-1} Cu ENM treatment). Further lesions in the anterior gills of crabs exposed to 1 mg L^{-1} Cu ENMs (panel c) included thickening of the epithelium, disruption of pillar cells, cells with eosinophilic inclusion bodies and increased cytoplasmic eosinophilia. However, the severest lesions were found in crabs from the 1 mg L^{-1} Cu as CuSO_4 treatment (panel d), which manifested as hyperplasia, hypertrophy, rupture of pillar cells, oedema and epithelium thickening. The annotations refer to; (rp) ruptured pillar cells; (eh) epithelial hyperplasia; (n) necrosis; (oe) oedema; (ch) congestion of haemocytes; (hpt) hypertrophy; (\blacktriangle) cells with eosinophilic inclusion bodies and increased cytoplasmic eosinophilia and (sm) swollen mucocytes. Sections were $7 \mu\text{m}$ thick and stained with haematoxylin and eosin.

for dissolved metals including Cu (Truchot and Rtal, 1998), and in the current study, exposure to CuSO_4 also resulted in Cu accumulation in this organ (Fig. 2c). A dose-dependent increase in hepatopancreatic Cu levels was seen in Cu ENMs exposed crabs by day 7, though the within-treatment concentrations did not increase significantly over time (Fig. 2c). The internal form of the metal was not known (i.e., whether remaining as a particle or dissolved metal in the tissue), but it is clear that exposure to Cu ENMs can elevate total metal concentrations in the internal organs. Some accumulation was also seen in the muscle of both Cu ENM and Cu salt exposed crabs by day 4, but the effect was transient in the Cu ENM treatments, unlike the muscle from the CuSO_4 exposed crabs where the tissue Cu concentration continued to rise (Fig. 2d). The posterior gill from at least the highest Cu ENM exposure showed increasing total Cu concentrations after day 4; tentatively suggesting that Cu from the ENM exposure was being redirected from the muscle to the gill. The dynamics need further investigation. Nonetheless, the Cu levels were much lower in the muscle tissue compared to the gills or hepatopancreas.

The Cu accumulation patterns were somewhat organ-specific, as there were no changes in the haemolymph and relatively low Cu accumulation in muscle, regardless of the form of the metal used for the exposures, or the exposure concentration (Fig. 2). These observations on accumulation can therefore only be explained by a regulated process that preferentially deposits Cu from either metal or nano exposure in the hepatopancreas. Both forms of the metal are theoretically possible, since the hepatopancreas is well-known for its role in processing particles as part of the normal biomineralisation processes of crustaceans (e.g., Hopkin and Nott, 1979), as well as dissolved metal. The absence of changes of total Cu in the haemolymph, despite elevations in the hepatopancreas, is also consistent with Cu homeostasis strategy of inert storage of metal-containing granules as part of an attempt to keep the body fluids constant.

4.3. Gill pathology and ionoregulatory status of the animals

Gill pathology arising from waterborne exposure to dissolved Cu is well documented in freshwater fishes (e.g., Al-Bairuty et al., 2013), and to a lesser extent in marine teleosts (Baker, 1969). Surprisingly, there are fewer detailed reports on the gill histopathology of shore crabs following dissolved Cu exposure in full strength seawater. In the present study, exposure to 1 mg L^{-1} of Cu as CuSO_4 for 7 days caused numerous morphological alterations to the lamellae of both the posterior and anterior gill filaments (Fig. 4 for anterior gill images). These included injuries associated with osmotic damage to the tissue such as hypertrophy of the epithelial cells and consequent changes in the extracellular space, separation of the epithelium at lamellar tips (i.e., possible oedema) with subsequent necrosis, as well as disruption of the structure of the pillar cells. The posterior gills showed more severe alterations than anterior gills with respect to injuries associated with loss of osmotic control (lamellar width, haemolymph space in the lamellae, Fig. 4) when exposed to CuSO_4 , possibly due to their functional differences, with the posterior gill tissue being the vulnerable osmoregulatory surface. However, all other aspects of pathology (e.g., congestion of gill, inflammation) were more severe in the anterior (respiratory) gill (see below). The extent of the gill injury with CuSO_4

(Fig. 4) is perhaps not surprising given the dose. Lawson et al. (1995) found that Cu concentrations as low as $50 \mu\text{g L}^{-1}$ caused ultrastructural changes in the apical membranes of the pavement epithelial cells and some loss of integrity of the pillar cells after 10 days. At ten-fold higher concentrations (0.5 mg L^{-1} Cu), Nonnette et al. (1993) reported extensive structural alterations involving hyperplasia of the epithelium, vacuolization, and necrosis after 5–6 days. Such observations are consistent with those reported here. The functional consequences of the gill injuries (Fig. 4) might include respiratory acidosis (Nonnette et al., 1993), but changes in the osmolarity and electrolyte concentrations in the haemolymph were not observed (Table 2). Weeks et al. (1993) also found that *C. maenas* showed no appreciable ionoregulatory disturbances after 7 days of exposure to 0.75 mg L^{-1} of Cu in 30 parts per thousand seawater.

Notably, the types of gill injury with exposure to 1 mg L^{-1} of Cu as the Cu ENM were broadly the same as CuSO_4 . There were no statistical differences in the severity of injuries associated with water balance (apparent oedema and sinusoid space, Fig. 4) between the 1 mg L^{-1} Cu ENMs and CuSO_4 . However, other injuries were more frequent in the animals exposed to the Cu ENMs (observed in all animals at the high Cu ENM concentration). There are currently no other reports of gill injury in *C. maenas* following exposure to Cu ENMs. Nonetheless, the injuries here are similar to those we have reported in the gills of freshwater-adapted rainbow trout (reactive hyperplasia, mild oedema, presence of a few necrotic cells) with the same Cu ENM (Al-Bairuty et al., 2013). In contrast to our previous work on fishes, the crabs here sometimes showed greater incidence of injury (individual pathologies in more animals within treatment) due to exposure to the nano form compared to the metal salt. This may be an organism-specific biological difference, as crustaceans already have the active biological machinery for forming mineral granules for inert metal storage, unlike trout that do this to only a minor extent with dietary Cu (Lanno et al., 1987). ENMs might interfere with this process, although any cross talk with naturally occurring biomineralisation processes in the tissue requires further investigation. In direct free ion toxicity arising from Cu dissolution at the gill surface to cause pathology from the ENM is very unlikely in the present study. The tissue Cu concentrations are not higher in animals exposed to the ENM form compared to those exposed to CuSO_4 (Fig. 2), and in any event the Cu dissolution is low/negligible from these Cu metal particles (see Shaw et al., 2012). Regardless, even if micromolar amounts of Cu were released at the gill surface, the Cu would easily be out-competed for binding at the gill surface by several hundred millimoles of Na ions in the seawater (Handy et al., 2002).

4.4. Oxidative stress and inflammation

Dissolved Cu is known to cause oxidative stress, either directly by catalysing the Haber-Weiss reaction in tissue leading to lipid peroxidation, or indirectly from systemic hypoxia associated with respiratory distress. For example, blue crab (*Callinectes sapidus*) fed 5 mg of Cu in the diet for 12 days showed lipid peroxidation and protein oxidation in the hepatopancreas, along with induction of glutathione peroxidase and catalase as enzymes involved in defending against oxidative stress (Brouwer and Brouwer, 1998). The inflammatory gill injuries reported here for the posterior gill were moderate in the ENM treatments and

more severe in the CuSO₄-exposed animals (Fig. 4). Although, acid-base parameters were not measured in the present study, some respiratory hypoxia is expected (see Nonnotte et al., 1993) from the level of gill pathology observed (Fig. 4).

Notably, for CuSO₄, a systemic inflammation response (i.e., due to lipid peroxidation/tissue injury) was evident from some congestion of the gill tissue with haemocytes, the presence of vacuoles in the hepatopancreas, and myofibril injury consistent with inflammation. Tissue inflammation associated with metal-induced oxidative stress is well-known (e.g., Cd in mussels, Sheir and Handy, 2010) and this may raise concerns about the immune health of the crabs. However, overall no changes were observed in TBARS and total GSH in crabs exposed to CuSO₄ compared to controls by the end of the experiment (Fig. 3). This implies the CuSO₄ exposure, for the animals that survived, was a manageable inflammation without loss of the first line of chemical antioxidant defence provided by the total glutathione. Moreover, while histological evidence of inflammation was present in the crabs exposed to the Cu ENMs, neither exposure concentration depleted total glutathione or caused any sustained increase of TBARS (Fig. 3). Thus overall, the hazard for oxidative stress with the Cu ENMs and CuSO₄ are very similar; despite the differences in gill injury. The mechanisms of ENM-exposure dependent inflammation need further exploration, particularly with regard to whether or not any internalised Cu particles (if present) were antigenic.

5. Conclusions

The current study provides some of the first data on Cu accumulation from waterborne exposure to Cu ENMs in comparison to Cu as CuSO₄ in the shore crab *Carcinus maenas*. The Cu accumulation pattern and biological effects of Cu as CuSO₄ were as expected for shore crabs in full strength seawater. A key question for environmental risk assessment is whether or not the nano form presents an additional hazard to the existing metal salt. In terms of lethal toxicity, the metal salt is more hazardous. However, for sub-lethal effects such as gill injury and inflammation, the ENM form may sometimes show a higher incidence of injury, depending on the precise endpoint used. The aetiology and incidence of injuries by type of material had some differences to our previous work on the same ENM in freshwater fish, where in every case the hazard of CuSO₄ was greater than the nano form for fish (Shaw et al., 2012; Al-Bairuty et al., 2013, 2016). There may be species of organism differences. Notably, the metal sequestration strategy of crustaceans should be considered more thoroughly for particle toxicology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aquatox.2017.08.006>.

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